Toxicity of Aluminum Silicates Used in Hemostatic Dressings Toward Human Umbilical Veins Endothelial Cells, HeLa Cells, and RAW267.4 Mouse Macrophages

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Background: Aluminum silicates have been used to control bleeding after severe traumatic injury. QuikClot (QC) was the first such product, and WoundStat (WS) is the most recent. We recently observed that WS caused vascular thrombosis when applied to stop bleeding. This study investigated the cellular toxicity of WS in different cell types that may be exposed to this mineral and compared the results with other minerals such as bentonite, kaolin, and QuikClot ACS+ (QC+).

Methods: Human umbilical vein endothelial cells (HUVEC), HeLa cells, and RAW267.4 mouse macrophage-like cells (RAW) were incubated directly with different concentrations of each mineral for 24 hours. Cell viability was determined metabolically using the AlamarBlue fluorescent technique. In another experiment, minerals were exposed to HUVEC via Transwell inserts with a polycarbonate filter (0.4-μm pore size) to prevent direct contact between cells and minerals for determining whether direct exposure or leaching compounds from minerals cause cytotoxicity.

Results: Incubation of HUVEC and RAW cells with 1 to 100 μg/mL of the minerals for 24 hours resulted in differential toxicities. The cytotoxicity of WS was equal to that of bentonite and higher than kaolin and QC+. Neither cell type survived for 24 hours in the presence of 100 μg/mL WS or bentonite. These minerals, however, had little effect on the viability of HeLa cells. In the second HUVEC experiment, a 10 times higher concentration of these compounds placed in Transwell inserts yielded no decrease in cell viability. This result indicates that leaching toxicants or binding of nutrients by the ion-exchange properties of minerals did not cause the toxicity.

Conclusions: Although aluminum silicates seem relatively innocuous to epithelial cells, all produced some toxicity toward endothelial cells and macrophages. WS and bentonite were significantly more toxic than kaolin and zeolite present in QC+, respectively, at equivalent doses. The cytotoxic effect seemed to be caused by the direct contact of the minerals with the cells present in wounds. These data suggest that the future clearance of mineral-based hemostatic agents should require more extensive cytotoxicity testing than the current Food and Drug Administration requirements.

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Several aluminum silicates derived from clay minerals have found application in the topical control of bleeding after severe traumatic injury. Dry mineral granules such as the original QuikClot (QC, Z-Medica Corporation, Wallingford, CT),[1] WoundStat (WS, TraumaCure, Bethesda, MD),[2,3] and a hemostatic gauze containing kaolin (Combat Gauze, CG, Z-Medica Corporation)[4] rapidly absorb the water in blood, concentrate the cells and clotting factors, and promote hemostasis. These agents may also act as physical barriers to bleeding from damaged blood vessels. QC composed of zeolite minerals was the first such product; and WS, described by the manufacturer as composed of smectite, is the most recent mineral-based hemostatic product cleared by the US Food and Drug Administration (FDA) for clinical application.

Clay minerals are fine-grained mineral aggregates of hydrous aluminum silicate particles that are usually formed by weathering or hydrothermal activity of volcanic rocks. They are defined by geologists by the size of the grains (1–2 μm) and have the unique property of being plastic when wet but firm when they become dry.[5] The activity of these materials to control bleeding is largely due to aluminum silicate structures that absorb relatively large amounts of water and activate the intrinsic (contact) clotting pathway.[6] Aluminum silicates exhibit a sheet-like organization and can be considered as charged particles with zones of positive and negative charges that interact with water to give them clay characteristics that account for their plasticity. These materials can be produced synthetically with greater control over composition and charge, but the exact composition of the materials used in the hemostatic products investigated here is not known.

Clay minerals are generally considered nontoxic to humans and have been widely used in cosmetics and as excipient in drugs and foods. Bentonite, which has a long history in pharmaceutical formulations,[7] along with kaolin are listed in the US Pharmacopeia.[8]

The sensitivity of some human cells to aluminum silicates was first reported by Murphy et al.[9] while investigating the possibility of aluminum toxicity on neuron cell cultures.

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Their study demonstrated a severe toxic effect (cell lysis) of clay minerals such as montmorillonite and bentonite toward human endothelial cells, but these compounds had little or no effect on neuroblastoma or oligodendroglial cells. In addition to cytotoxicity, application of large amounts of granular/powder material, in the form of QC, CG, or WS, to a bleeding wound with major vascular injuries poses the potential risk of systemic embolism. Our initial evaluation of WS in a pig extremity wound model showed that microscopic particles of WS penetrated and remained in the injured vessels even after extensive wound debridement.[4] A follow-up study in a pig neck wound model to further define the toxicity of this product revealed vessel necrosis and occlusive thrombi formation in the WS-treated vessels (both in artery and vein) 2 hours after repair and blood reflow.[10] This study investigated the in vitro toxicity of WS, bentonite, kaolin (a mineral component of CG), and the new formulation of QC (QuikClot ACS+ [QC+]) toward HUVEC, HeLa cells derived from a cervical carcinoma, and RAW264.7 mouse macrophage-like cells (RAW), a cell line often used for in vitro screening of biomaterials for potential production of inflammatory mediators.[11,12]

MATERIALS AND METHODS

Materials

WS was purchased from TraumaCure (Bethesda, MD), bentonite and kaolin from Sigma-Aldrich Chemical Co. (St. Louis, MO), and QC+ from Z-Medica. QC+ was ground to a fine powder in a ball mill until the particles were about 1 to 2 μm in size. One gram of each mineral was placed in a glass vial that was then autoclaved to sterilize the material. Ten milliliters of sterile phosphate-buffered saline was added to each mineral, and the suspension was further diluted in different volumes of tissue culture medium to achieve final concentrations of 1 to 100 μg/mL.

Cell Culture

First passage HUVEC (Lifeline Cell Technology, Walkersville, MD) were cultivated on 1% gelatin-coated 75-cm^2 culture flasks (Corning Incorporated, Corning, NY) containing complete Vasculife VEGF Medium supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and Fungizone (0.25 μg/mL; Invitrogen Corporation, Carlsbad, CA). The cells were cultivated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2) with medium changes every 2 days until confluence. Before an experiment, HUVEC were subcultivated with Trypsin/ethylenediamine tetraacetic acid onto 1% gelatin-coated Costar 48-well multiplates (Corning Incorporated, Corning, NY) at 5,000 cells/cm^2, grown to confluence, and kept for 72 hours to produce a postmitotic cell layer. On the day before the experiment, the medium was changed. Only the second through the fifth population doublings of cells were used. HeLa cells and RAW cells were obtained from the American Type Culture Collection (Manassas, VA) and cultivated in advanced Dulbecco's modified Eagle's medium (Invitrogen) with 5% FetalClone III calf serum (Fisher Scientific, Houston, TX) and seeded into 48-well multiplates at 10,000 cells/cm^2 and used at 80% confluence. Two hundred microliters of culture media containing different concentrations (1–100 μg/mL) of each mineral was added directly to the cells, and the plates were incubated at 37°C for 24 hours in a humidified incubator with 95% air and 5% CO_2.

To determine the importance of physical contact between minerals and cells for inducing toxicity, the minerals were suspended in the culture media (indirect exposure) in a separate experiment. Confluent HUVEC were seeded into 24-well multiplates, and Transwell inserts (Corning Life Sciences, Corning, NY) with polycarbonate filters (0.4-μm pore size) were placed over the cell layer in each well. One hundred microliters of stock mineral suspension in phosphate-buffered saline (1 mg/mL) were added to each chamber and incubated as before.

Cell Viability

Cell viability was assessed 24 hours after mineral treatment using AlamarBlue (Biosource International, Camarillo, CA), which is converted to a fluorescent compound in amounts proportional to the number of viable cells. The assay is based on the metabolic activity of living cells, which converts a redox dye (resazurin) into a fluorescent end product (resorufin). The cells were incubated for 2 hours at 37°C with a culture medium containing 10% AlamarBlue. After incubation, fluorescence was measured at 545-nm excitation and 590-nm emission wavelengths using a SpectraMAX M2 microplate reader (Molecular Devices, Sunnyvale, CA). All cells were regularly observed under phase contrast microscopy. Nonviable or damaged cells rapidly lose their metabolic capacity and thus generate a lower fluorescent signal. To obtain some idea about timing and mode of cell death, the Live/Dead assay (Invitrogen, Carlsbad, CA) based on the manufacturer's instruction was performed on HUVEC. Live/Dead phase contrast and fluorescence images captured from an Olympus FluoView 10 confocal microscope (Olympus, Center Valley, PA) were merged to produce the resulting images.

Data Analysis

Data are presented as the mean ± SEM. Each experiment was repeated three times. The data point represents quadruplicate measurement of cell viability for each concentration performed in a 48-well multiplate. Differences between or among the groups were analyzed by using the independent samples *t* test or one-way analysis of variance combined with Tukey (equal variances assumed) test or Games-Howell (equal variances not assumed) posttest through SPSS statistical software. A difference of $p < 0.05$ was considered significant.

RESULTS

Figure 1 illustrates the differential effects of WS, bentonite, kaolin, and QC+ on HUVEC viability. Direct exposure of WS or bentonite to these cells resulted in dose-dependent cell death, which was more severe than exposure to kaolin or QC+. The difference in toxicity became significant ($p < 0.05$) at 25 μg/mL and higher concentration levels. Some of the HUVECs survived the 100 μg/mL dose of kaolin or QC+ for 24 hours, but none survived incubation with WS or bentonite.

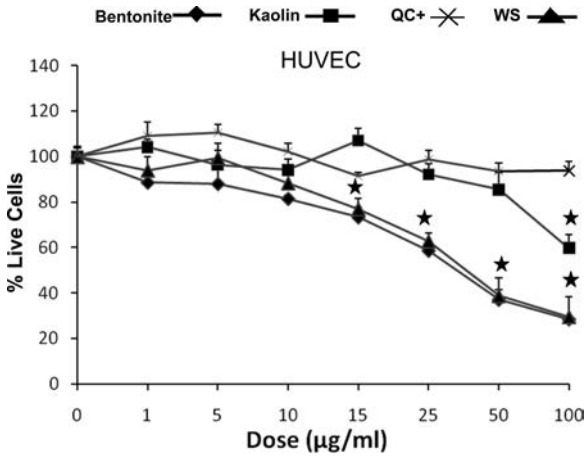

Figure 1. Toxicity of aluminum silicates on HUVEC viability 24 hours after incubation. The 20% apparent cell survival in WS and bentonite groups is an assay artifact. Values are presented as means ± SEM (n = 12). HUVEC, human umbilical vein endothelial cells. *$p < 0.05$ versus baseline (0 mg/mL concentration).

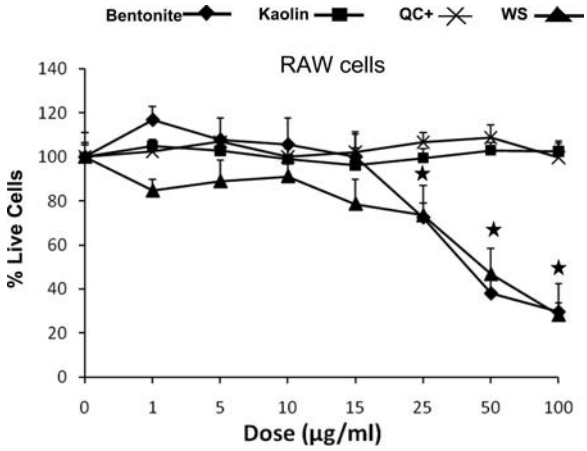

Figure 2. Toxicity of aluminum silicates on mouse macrophage-like cell (RAW) viability 24 hours after incubation. WS and bentonite were the only minerals that had toxic effects on these cells. Values are presented as means ± SEM (n = 12). *$p < 0.05$ versus baseline (0 mg/mL concentration).

Although kaolin was found to be less toxic than WS or bentonite, it still resulted in more than 60% HUVEC death at a 100 µg/mL concentration. The apparent viability of about 20% of the WS-treated cells at the higher doses is due to reduction of dye by material released by dying cells (background activity). Direct observation of cell cultures confirmed total cell lysis in those experiments.

Similar results were also obtained when the aluminum silicates were incubated with RAW cells as seen in Figure 2. The only difference was that, even at the highest concentration, kaolin had no toxic effect on these cells. In contrast to HUVEC and RAW cells, the silicate compounds, even at 100 µg/mL concentration, had little or no effect on the viability of HeLa cells (Fig. 3). Whether these results are due to the fact

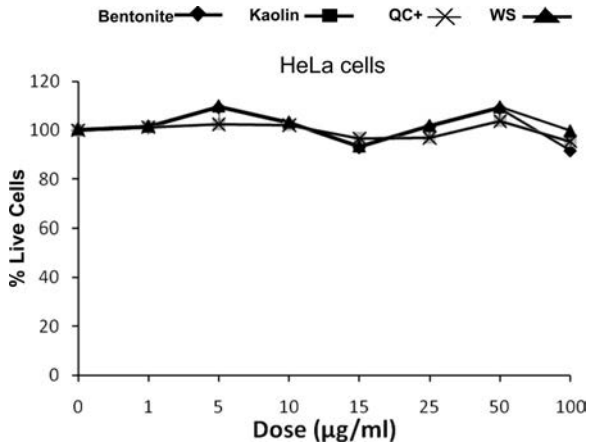

Figure 3. Toxicity of aluminum silicates toward HeLa cells, an epithelial cell type. No significant reduction in viability was observed 24 hours after incubation at the tested doses. Values are presented as means ± SEM (n = 12).

that HeLa cells are a transformed, immortal cell line well adapted to cell culture or related to their epithelial origin is unknown, but none of these compounds affected the viability or growth of this cell type at these concentrations.

Finally, when the silicates were placed in Transwell filter inserts with 0.4-µm pore size and suspended over HUVEC at 10 times the highest concentration that was used above (1 mg/mL), no significant change was seen in viability of the cells up to 48 hours after incubation. The 0.4-µm pore size was considerably smaller than the size of the aluminum silicate particles (1–2 µm) and prevented direct contact of minerals with the cell layer. This result excluded the possibilities of leaching toxins from the minerals or their ability to remove important nutrients because of their known ion-exchange properties as the cause of toxicity; therefore, direct contact between the mineral and the cell seemed necessary for cytotoxicity effect.

HUVEC observation with phase contrast microscopy (Fig. 4) showed rapid transformation (within 4 hours) of live (Fig. 4, A) to dead cells (Fig. 4, B) when HUVEC were exposed to WS or bentonite and minerals accumulated over the cell surface (black arrows) as if the minerals were attracted to the cell membrane. Pyknotic nuclei were observed by this time (white arrows), and blebbing of some cells was evident, indicating oncotic cell death.[13] Under confocal microscopy (Fig. 5), untreated HUVEC demonstrate the green appearance of living cells (Fig. 5, A). The WS-treated HUVEC (Fig. 5, B) retain sufficient membrane integrity to contain the calcein green dye, but the early stages of oncotic cell death are evident by blebbing of the cell surface (arrows) after 3 hours of incubation. The inset picture (upper right corner) shows ethidium homodimer-positive red nuclei, indicating dead cells. In no cases did the observed cellular and/or nuclear fragmentation indicate apoptotic cell death; i.e., mineral-induced cytotoxicity occurred rapidly and was not initiated by the responding cell.

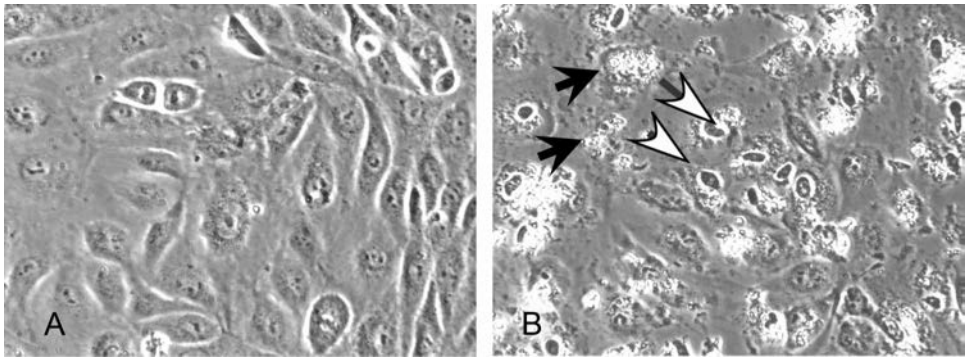

Figure 4. Phase contrast micrographs of WS-treated HUVEC. Photographs of culture plates of a control monolayer of HUVEC (*A*) and a similar monolayer 4 hours after the addition of 50 μg/mL of WS (*B*). Black and white arrows denote accumulation of mineral and pyknotic nuclei, respectively.

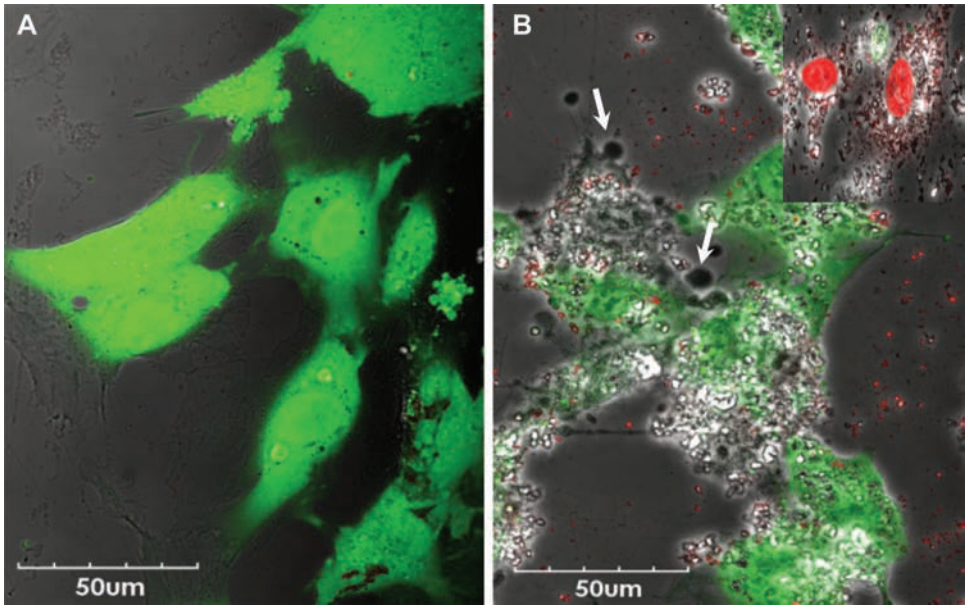

Figure 5. Merged phase contrast and fluorescent confocal acquired images of HUVEC after exposure to WS by the Live/Dead assay. (*A*) Appearance of untreated living cells (green). (*B*) Some WS-treated HUVEC still possessing sufficient membrane integrity to contain the calcein green dye but the early stages of oncotic cell death are evident by blebbing of the cell surface (arrows). Inset picture shows ethidium homodimer-positive red nuclei, indicating dead cells.

DISCUSSION

The results of this study indicate that some FDA-cleared mineral-based hemostatic products are not innocuous, and this finding should be part of the benefit/risk evaluation before their use is considered. Addition of 100 μg/mL concentration of the minerals to the cells in this in vitro study is most likely much less than the amount of the minerals applied in vivo. For example, pouring of a bag of WS (150 g smectite) into an average size wound with 200 mL blood pool could result in WS concentration of 750 mg/mL, or in the case of each CG that is coated with ~1.5 g kaolin (10% of the weight), the concentration of kaolin may reach 2.5 mg/mL in the wound, assuming that one-third of kaolin is washed out from the gauze and suspended in the blood. These minerals seem to be relatively safe to epithelial cells of the skin and the inner digestive tract and are used in cosmetics and pharmaceutical products. Some, however, are particularly toxic to certain cell types, such as endothelial cells and macrophages that are present in wounds. It should also be mentioned that the toxicity of an agent such as WS becomes apparent in some cells as early as a 30 minutes after exposure to endothelial cells at the 100 μg/mL concentration, a time interval that is consistent with indicated temporary use of this agent for hemorrhage control.

The results reported here are consistent with those reported by Murphy et al.[9] that earlier identified a marked sensitivity of endothelial cells to several aluminum silicate minerals. Both kaolin and the zeolite in QC+ seem to be less toxic than WS and bentonite. Given the similarities in particle size, color of the dry material, and cytotoxicity, WS may be composed primarily of bentonite. A principal difference between bentonite and kaolin is the ion-exchange capacity of

each mineral: bentonite, ~180 mEq; kaolin ~4 mEq. This capacity is related to the sodium, calcium, and magnesium content of each mineral; but other elements may also play a role. Whether lower cytotoxicity of kaolin is related to its low ion-exchange capacity or whether kaolin and zeolites have fewer impurities is currently unknown.

The in vitro cytotoxicity data found here were consistent with the in vivo findings in experimental safety studies in swine.[10,14] A 2-hour WS treatment of an external wound in pigs to control arterial and venous bleeding caused significant endothelial damage and led to occlusive thrombosis in the majority of treated vessels within 2 hours after blood reflow.[10] The toxicity of WS and bentonite is the result of direct contact of the minerals with the sensitive cells rather than chemical compounds that may be washed out from the agents and affect the cells. A 10-fold higher amount of WS added to the cell culture medium had no detrimental effects on HUVEC viability as long as the materials were physically separated from the cells. Interestingly, this result was also in agreement with the in vivo finding in the swine study. In a few experiments in which WS and blood were poured into a wound with isolated but uninjured vessels (no direct contact between WS and endothelium), the endothelial layer remained intact; and none of the vessels developed thrombosis. The damage to adventitia (delamination), however, was still present as a result of exposure to WS. Because it appears that direct contact of minerals is the main cause of cytotoxicity, any means to limit contact of the hemostatic minerals with tissues without affecting efficacy may reduce toxicity of these products. This approach was successfully implemented with respect to the original QC, a granular hemostatic agent with significant exothermic reaction on hydration. To prevent burning injuries, the original materials were packaged in small porous bags to avoid direct contact of the granules with the tissues (QuikClot ACS). Later on, the zeolite formulation was also modified to create a new product (QC+), which has little or no exothermic reaction when applied to a bleeding wound.[13] Although others have shown successful results with QC+,[15] this product has not been effective against arterial bleeding in our experimental hemorrhage model.[16]

The chemical composition of the minerals tested in this study and how they relate to their hemostatic activities have been reported. Zeolites are crystalline hydrated aluminosilicate minerals consisting of interlocking tetrahedrons of SiO_4 and AlO_4 with microporous structure that are negatively charged, attracting a wide variety of positive ions such as Na^+, K^+, Ca^+, and Mg^+. These positive ions are loosely held and can readily exchange for others, permitting ion exchange and water absorption. Zeolites are known as molecular sieves with pore size of 6 Å in the dehydrated form. The chemical formula of a Zeolite example (natrolite) is $Na_2Al_2Si_3O_{10}{\cdot}2H_2O$.[17] The hemostatic activity of dehydrated form of zeolite (QC) is attributed to its high capacity to absorb water from the blood and/or release of heat on contact with blood. The reactions concentrate clotting proteins and cells in the wound and promote coagulation.[18] The new QC, QC ACS+, consists of synthetic zeolite beads with proprietary formula that generates minimum heat when mixed with blood and rehydrated. Hemostatic activity of the QC ACS+ was shown to be equal to original QC granules.[19]

WS (smectite), bentonite (a smectite and predominant component of WS), and kaolin (active component of CG) all belong to layered clay mineral groups. Clay minerals are hydrous aluminum phyllosilicates with variable amounts of iron, magnesium, alkali metals, alkaline earths, and other cations.[20] The smectites are three-layer clay minerals with crystallites of <2 μm in diameter. They consist of an octahedral alumina sheet sandwiched by two sheets of tetrahedral silica, which readily absorb water and swell on hydration, thereby thickening the blood and facilitating clot formation. Smectites have a net negative surface charge, which serves as a platform for activating proteins, including activation of the contact pathway of coagulation.[21] This group of minerals had the highest toxicity toward endothelial and macrophage-like cells. WS contains bentonite and other smectite minerals, but the exact composition of materials used for producing the hemostatic agent is unknown.

Kaolin is a two-layer clay mineral with the chemical composition $Al_2Si_2O_5(OH)_4$. It consists of a tetrahedral sheet of silicate bonded through oxygen atoms to an octahedral sheet of alumina with oxygen and hydroxyl group attachments.[22] This structure gives kaolin a strong negative surface charge at normal blood pH (7.4), which activates factor XII efficiently and initiates intrinsic clotting cascade.[23] In comparison with three-layer clays (WS), kaolin toxicity was mild and seen only at higher concentrations on the more sensitive (endothelium) cell type. Detailed chemical studies are required to correlate the chemical composition and the physical properties of the minerals with their biological toxicity to better understand the mechanism of cell injuries by these minerals.

The current mineral-based hemostatic products mentioned above have received Premarket Notification 510(k) by the FDA as medical devices for temporary control of bleeding in external wounds.[24] This pathway requires some standard safety testing, including in vitro cytotoxicity with fibroblasts and in vivo sensitivity, irritability, and systemic toxicity, which are done generally in small animals. These tests evaluate the potentially adverse effects of chemicals that may be eluted from a medical device. Although no reaction indicates that a material is free of harmful extractables, it is certainly not evidence that the device is fully biocompatible and safe for any indications.[25] Considering that hemostatic devices are intended to stopping bleeding from vascular injuries, there is a likelihood that they will come in direct contact with endothelial cells in the injured vessels, as well as macrophages drawn to the site of injury. Our results indicate that the current level of toxicity testing to achieve FDA 510(k)[17] clearance for mineral-based hemostatic products is inadequate. More extensive cytotoxic testing relevant to the agents' indications should be required before such products are cleared for clinical use.

In conclusion, WS and bentonite showed a high degree of toxicity to endothelial cells and macrophages, whereas kaolin and zeolite were less toxic. The toxic effects (cell death) of these agents required direct contact with the cells,

which is consistent with the ways these agents will be used clinically to control hemorrhage. Future licensing of mineral-based hemostatic agents for clinical use should require more extensive cytotoxicity testing than those currently required by the FDA.

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